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Substrate-Assisted Catalysis Unifies Two Families of Chitinolytic Enzymes

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Abstract: Hen egg-white lysozyme has long been the paradigm for enzymatic glycosyl hydrolysis with retention of configuration, with a protonated carboxylic acid and a deprotonated carboxylate participating in general acid−base catalysis. In marked contrast, the retaining chitin degrading enzymes from glycosyl hydrolase families 18 and 20 all have a single glutamic acid as the catalytic acid but lack a nucleophile on the enzyme. Both families have a catalytic $(\beta\alpha)_8$-barrel domain in common. X-ray structures of three different chitinolytic enzymes complexed with substrates or inhibitors identify a retaining mechanism involving a protein acid and the carbonyl oxygen atom of the substrate's C2 *N*-acetyl group as the nucleophile. These studies unambiguously demonstrate the distortion of the sugar ring toward a sofa conformation, long postulated as being close to that of the transition state in glycosyl hydrolysis.

Introduction

Lysozymes and chitinases hydrolyze C2 *N*-acetylated $\beta(1-4)$-linked substrates such as chitin and peptidoglycan. The catalytic mechanism of hen egg-white lysozyme is understood in good detail.[1,2] The concerted action of two acidic residues, the general acid−base Glu35 and the nucleophile Asp52, results in a reaction with retention of the configuration at the anomeric carbon atom. In the first reaction step, Glu35 acts as a general acid and protonates the glycosidic oxygen of the scissile bond, leading to bond cleavage and formation of a positively charged oxocarbonium intermediate at the −1 sugar residue (Figure 1a; nomenclature of saccharide binding sites as proposed by Davies et al.,[3] Table 1). Asp52 is located on the opposite side of the scissile bond and has been proposed to either stabilize the oxocarbonium intermediate via electrostatic interactions[2,4] or form, via an oxocarbonium ion-like transition state, a covalent intermediate with a bond to the −1 C1 atom.[5] In the next steps, the leaving group diffuses out of the active site and is replaced by a nucleophilic water molecule. This water molecule, assisted by the negatively charged Glu35 now acting as a general base, then attacks the intermediate to complete the reaction. As the water molecule attacks from the same side from where the leaving group has left, the configuration at the anomeric carbon atom is retained. Stabilization of the oxocarbonium ion is a fundamental prerequisite of the mechanism since oxocarbonium ions have extremely short lifetimes in solution.[6]

In contrast, chitinases, chitobiases, and related enzymes from glycosyl hydrolase families 18 and 20,[7,8] which act also with retention of configuration,[9,10] have only a single glutamic acid in their active sites but no aspartate.[11−15] The two families share no obvious sequence homology but have a very similar $(\beta\alpha)_8$ barrel catalytic domain with the catalytic Glu in equivalent positions at the C-terminal end of the fourth β-strand of the barrel (Figure 2a,b). Based on the detailed analysis of two complexes previously described by us[9,15] and the new X-ray structures of chitotetraose complexed to hevamine, and chitobiose bound to Chitinase A, we show that in families 18 and 20 participation of the carbonyl oxygen atom of the substrate's C2 *N*-acetyl group in the reaction provides the required stabilization of the oxocarbonium ion (Figure 1b). In addition, our studies demonstrate the distortion of the −1 sugar ring toward a sofa conformation.

Results

The 3-D structures of enzyme/chitin oligomer complexes are listed in Table 1. Hevamine, a plant chitinase-lysozyme from *Hevea brasiliensis*, and the bacterial Chitinase A from *Serratia marcescens* both belong to glycosyl hydrolase family 18. In

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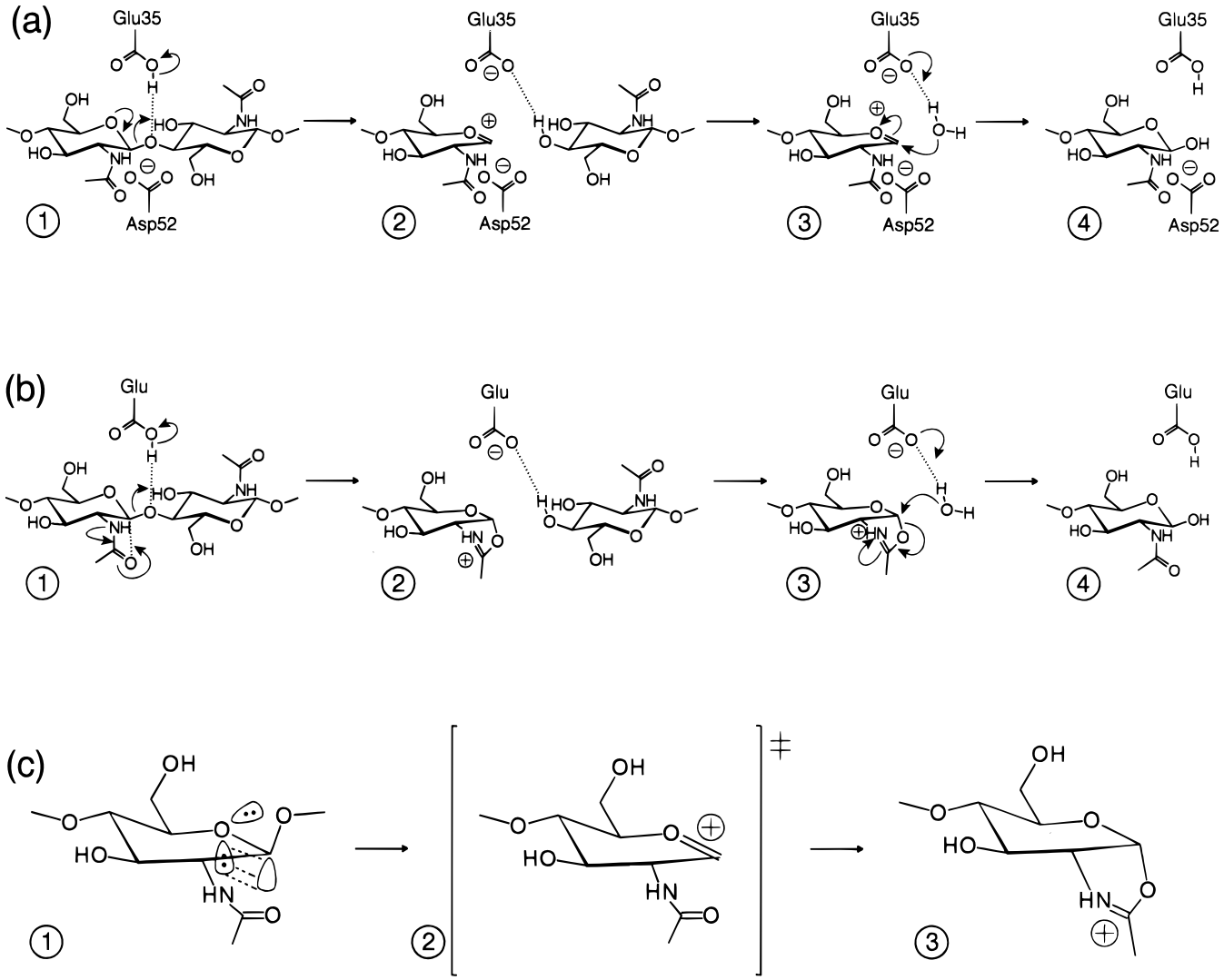

Figure 1. (a) Glycosyl hydrolysis in hen egg-white lysozyme following general acid−base catalysis. Glu35 is the acid protonating the glycosidic oxygen and Asp52 is the nucleophile proposed to stabilize the reaction intermediate electrostatically.[4] The two enzymatic carboxylates have respective pK_a's of 6 and 4.[1,2] (b) Scheme for retaining chitinolytic enzymes where stabilization is proposed to occur via a covalent oxazolinium ion intermediate. The nucleophile is the *N*-acetyl group of the substrate itself. For stereochemical reasons, this scheme is limited to retention. (c) Nature of the distorted substrate with its orbital geometry, the putative oxocarbonium transition state, and the proposed reaction intermediate.

the crystal chitotetraose binds at sites −4 to −1 in the active site of hevamine, and chitobiose binds at sites +1 and +2 in Chitinase A. Superposition of the catalytic domains of these enzymes positions the O1 atom of the −1 sugar in hevamine and the O4 atom of the +1 sugar in Chitinase A 1.8 Å apart (Figure 2a); they would be the same atom in an intact substrate. The directions of the C1−O1 bond of the −1 sugar and the C4−O4 bond in the +1 sugar are not appropriate for direct bond formation, and hence, it is not possible simply to link the chitotetraose and chitobiose into a single continuous chain across the scissile bond.

In contrast, an intact glycosidic bond across the cleavage site between residues −1 and +1 is seen in the crystal structure of *S. marcescens* chitobiase, a glycosyl hydrolase family 20 enzyme, complexed with the disaccharide chitobiose[15] (Figure 2b). The presence of an intact substrate bound in the active site of this enzyme may result from the high salt concentration used for crystallization along with restricted flexibility of the enzyme imposed by crystal packing.[15] The +1 sugar has a normal $^4C_1$ conformation, but the −1 sugar is distorted toward a 4-sofa conformation, with the glycosidic oxygen in a nearly axial position. This conformation, with the C1, C2, C3, C5, and O5 atoms nearly coplanar, is comparable to the postulated planar oxocarbonium ion intermediate in lysozyme[16] (Figure 1c).

Assuming a similar distortion of the −1 sugar occurs in family 18 chitinases, the chitotetraose bound at sites −4 through −1 in hevamine and the chitobiose complexed to Chitinase A at sites +1 and +2 can be linked into a continuous chain across the scissile bond. Indeed a covalent link between the −1 and +1 sugars is only possible if the −1 sugar is distorted toward the 4-sofa conformation observed in chitobiose bound to chitobiase. A hexasaccharide can be modeled in this way in the active site of family 18 Chitinase A, without requiring a change of conformation and position for the carbohydrate residues of sites −4, −3, −2, +1, and +2 (Figure 2c,d).

Several enzyme−substrate interactions valid for the modeled hexasaccharide are directly derived from the complexes. The planes of the −1 and +1 sugar rings are roughly perpendicular to one another, and the H-bond between the O3 of the +1 sugar with O5 of the −1 sugar residue is broken. This gives φ and ψ angles of the glycosidic linkage[17] in chitobiase of −45° and

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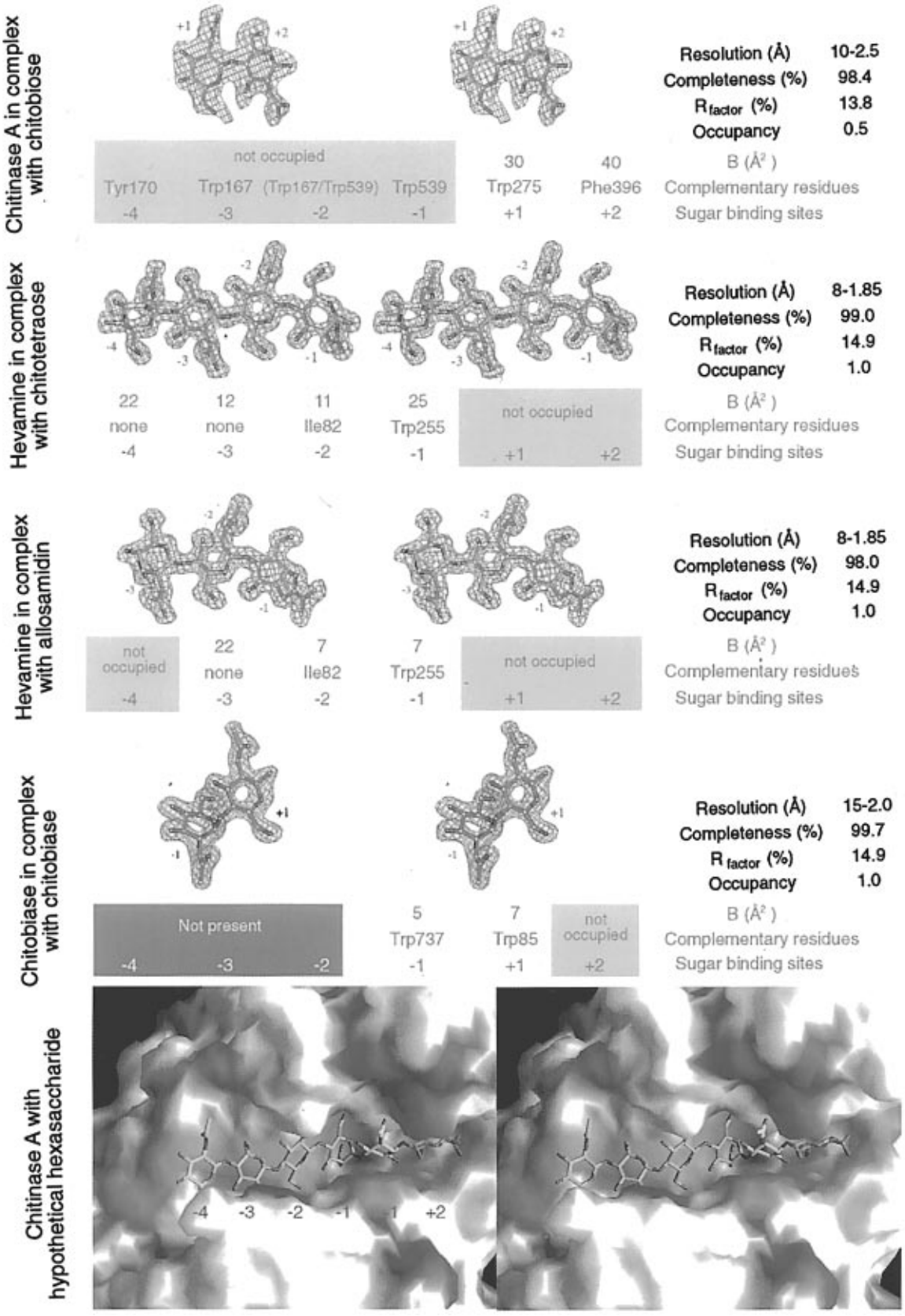
Table 1. Enzyme−Substrate/Inhibitor Complexes for Hevamine and Chitinase A (Glycosyl Hydrolase Family 18) and Chitobiase (Family 20)[a]



[a] At the top, the sugar binding sites are shown, numbered from −4 to +2 with the scissile bond between moieties −1/+1. For each individual saccharide residue bound in the complexes, the mean temperature factor is given, together with the amino acid residues providing complementary surfaces to the sugar rings. In addition, stereograms with refined $2F_o - F_c$ electron density around the saccharides are shown for each of the complexes. Crystallographic details characterizing the complexes are listed on the right. Chitinases of family 18 are assumed to possess at least six subsites, where family 20 has no site beyond −1 as the active site is a pocket rather than a groove. The surface representation (in stereo) shows our modeled hexasaccharide in Chitinase A, with the aromatic residues in blue and the aliphatic hydrophobic residues in green. Residues −4/−3/−2 of the constructed hexasaccharide (pink) have been taken from the hevamine−chitotetraose complex, residues −1/+1 (yellow) are from the chitobiase−chitobiose complex, and residue +2 (purple) is from the complex of Chitinase A with chitobiose.

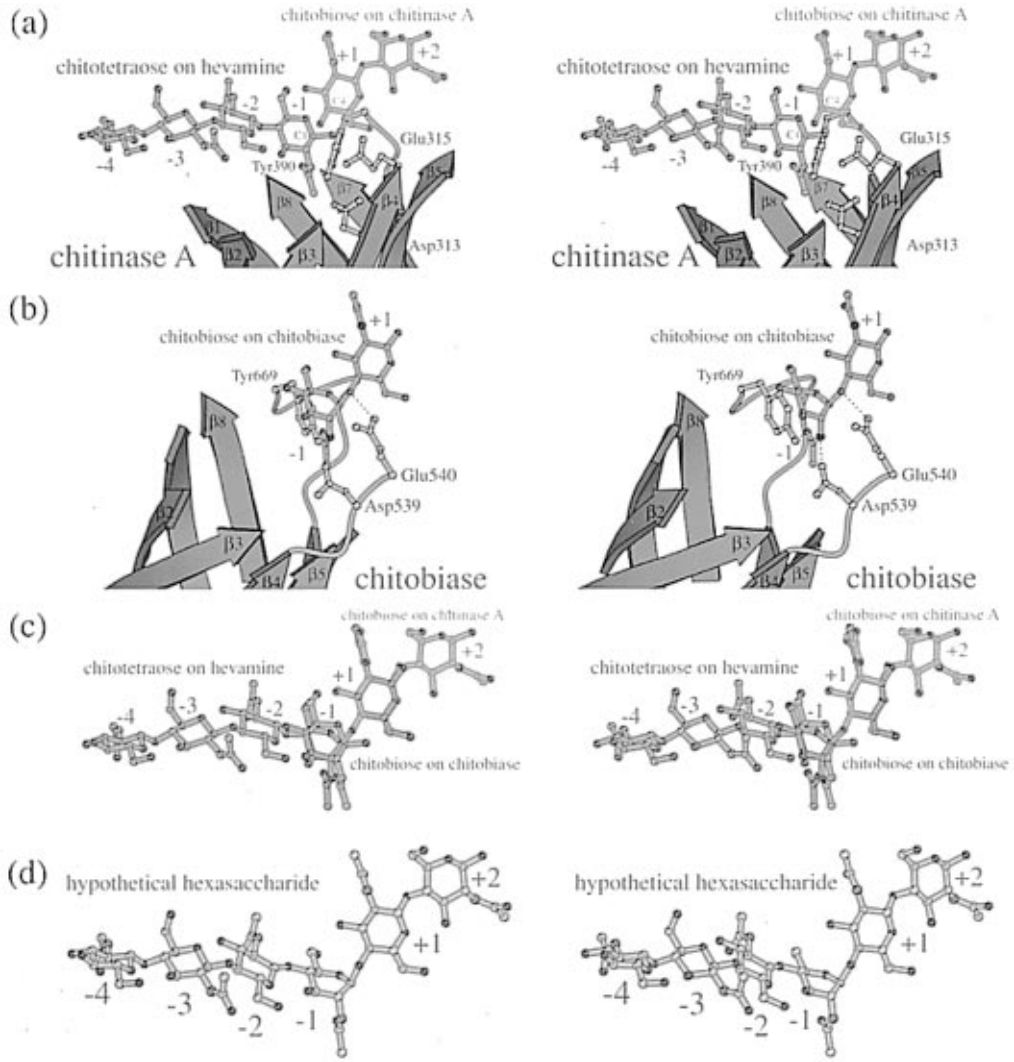


Figure 2. (a) Comparison of bound oligosaccharides after superposition of the catalytic domains of family 18 Chitinase A and hevamine. The central core of the $(\beta\alpha)_8$-barrel of Chitinase A is shown in green. Chitotetraose binds at sites −4 to −1 in hevamine (brown) and chitobiose at sites +1 and +2 in Chitinase A (pink). The two cannot be linked into a continuous chain without distorting the −1 sugar. (b) Chitobiose bound with an intact scissile bond at −1/+1 in chitobiase, a family 20 enzyme. The enzymes in parts a and b are not homologous, and the barrel axes do not align when the common substrate atoms are superimposed. (c) Superposition of the oligosaccharides shown in parts a and b, based on the position of the +1 sugar. A continuous chain across the scissile bond of the sugars in part a can only be formed using the conformation of −1 from part b. (d) Hexasaccharide obtained from the superposition in part c after energy minimization in the presence of Chitinase A provides a model of the intact oligosaccharide substrate in the Chitinase active site. Capturing such a complex has not been possible experimentally.

+137°, instead of the +90° and +100° angles observed in an extended chitin chain. All other oxygen and nitrogen atoms of the −1 sugar make H-bonds to the enzyme. The proton-donating catalytic acid (Glu540 in chitobiase, Glu127 in hevamine, and Glu315 in Chitinase A) is within H-bonding distance of the glycosidic oxygen of the scissile bond[15] (or the equivalent O1/O4). The binding of the −1 sugar *N*-acetyl group in chitobiase is characterized by polar interactions of its amide nitrogen with Asp539, its carbonyl oxygen with the hydroxyl group of Tyr669, and by hydrophobic interactions of its methyl group with Trp616 and Trp639. In hevamine Asp125, Tyr183 and Tyr6 are the corresponding amino acids forming similar interactions (Figure 3). The conformation of the *N*-acetyl group is such that its carbonyl oxygen points toward C1 of the −1 sugar, with the C1−C2−N2−C7−O7 atoms nearly in one plane and with an O7−C1 distance of 3.0 Å for chitobiase and 2.8 Å for hevamine.

(17) IUPAC−IUB Joint Commission on Biochemical Nomenclature *Eur. J. Biochem.* **1983**, *131*, 5−7.

Discussion

The extensive interactions of the C2 *N*-acetyl group and the position of its carbonyl oxygen atom strongly suggest a catalytic role for this group. Solution studies have shown that a C2 *N*-acetyl group enhances spontaneous hydrolysis of methyl-β-D-glucopyranoside more than 1000-fold.[18] An appropriately oriented *N*-acetyl group with its carbonyl oxygen in close proximity to C1 might also enhance enzyme-catalyzed carbohydrate hydrolysis. This could be either through electrostatic stabilization of a positively charged oxocarbonium ion intermediate, similar to the putative role of Asp52 in lysozyme,[19] or by forming an oxazolinium ion intermediate with a covalent bond between the *N*-acetyl carbonyl oxygen and the C1 atom (Figure 1b). Subsequently this reaction intermediate can be hydrolyzed by an incoming water molecule. An oxazolinium ion intermediate is an attractive alternative to electrostatic

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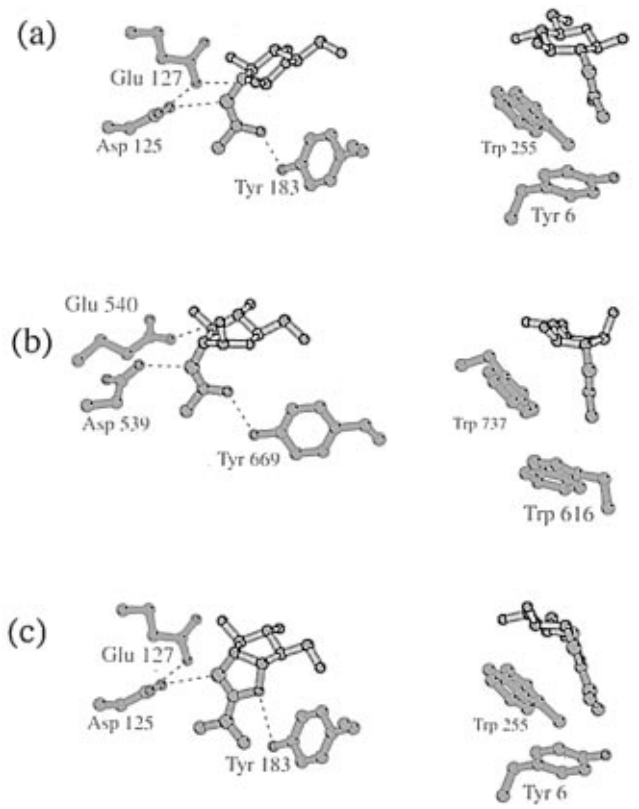

Figure 3. Observed interactions, in two orientations, of the −1 sugar in the three complexes of (a) hevamine with chitotetraose, (b) chitobiase with chitobiose, and (c) hevamine with allosamidin. The sketch shows from top to bottom the binding mode of the $^4C_1$ chair, the 4-sofa, and the covalent oxazolinium ion mimicked by allosamidine. The catalytic residue (Glu127 in hevamine, Glu540 in chitobiose) is shown in green. The binding of the *N*-acetyl group (brown) is characterized by polar interactions of its amide nitrogen to an aspartate (red), its carbonyl oxygen to the hydroxyl group of a tyrosine (red), and hydrophobic interactions of its methyl group with tryptophans/tyrosines (red). In these nonhomologous enzymes, residues performing similar functions sit on different strands of the central β-barrel.

stabilization as the positive charge can be delocalized over a larger volume. Support for a covalent oxazolinium ion intermediate comes from a recently published synthetic method to produce high molecular weight chitin from a chitobiose oxazoline derivative in the presence of *Bacillus* chitinase.[20] In addition, a thiazoline derivative of *N*-acetylglucosamine, in which the oxygen in the oxazoline ring has been replaced by a sulfur,[21] is a potent competitive inhibitor of jack bean *N*-acetylhexosaminidase, another family 20 enzyme, binding 20 000 times more tightly than *N*-acetylglucosamine.

Structural evidence for a covalent intermediate comes from the natural chitinase inhibitor allosamidin[9] (Table 1 and Figure 3c). Allosamidin binds with high affinity to family 18 chitinases but not to HEWL. It is similar to *N*-acetylglucosamine but lacks a pyranose ring oxygen and contains an oxazoline ring in which the methyl group is substituted by dimethylamine. This stabilizes the C1−O7 bond as the dimethylamine group has a higher electronegativity than the methyl group and as there is no glycosidic ring oxygen for lone pair donation (see below). Allosamidin thus exerts its inhibitory effect by acting as a nonhydrolyzable analogue of the oxazolinium ion intermediate.

From our enzyme−substrate complexes four connected factors stand out that are important for the catalytic mechanism of family 18 and 20 chitinolytic enzymes. These are the relative orientations of the sugars in sites −1 and +1, the absence of H-bonds to O5, the distorted conformation of the −1 sugar, and the special conformation of the *N*-acetyl group. The roughly perpendicular orientations of the −1 and +1 sugars prevent a hydrogen-bonding interaction between the O3 of the +1 and the O5 of the −1 sugar residue. The absence of this H-bond will facilitate departure of the leaving group. Moreover, in a sugar distorted toward a 4-sofa conformation, the antibonding orbital of the scissile bond is in a favorable position to overlap with the nonbonding orbital containing one of the lone pairs of O5 (Figure 1c). Lone pair donation by O5 to the antibonding orbital of the scissile bond accelerates the expulsion of the leaving group,[22] and the absence of an H-bond to O5 further enhances the degree of lone pair donation. These first three factors are equally valid for lysozyme and other retaining glycosidases acting on β-linked saccharides. However, in the chitinolytic enzymes of families 18 and 20, the special orientation of the *N*-acetyl carbonyl oxygen atom allows stabilization of the reaction intermediate and a covalent oxazolinium ion intermediate can be formed. It is an intriguing possibility that even HEWL might utilize the C2 *N*-acetyl group to assist hydrolysis of peptidoglycan and chitin oligosaccharides. In agreement with such a notion is the recent finding that HEWL exclusively cleaves after an *N*-acetylglucosamine residue in 32% deacetylated chitosan.[23] It would be an exciting experiment to bind a substrate analogue across the scissile bond in HEWL.

Conclusions

Our results show how substrate is bound in the active site of chitinolytic enzymes and that distortion is required. Substrate distortion was initially postulated for lysozyme,[16] and some structural evidence has been provided.[2] However, the distortion that we observed for an intact true substrate bound across the cleavage site in chitobiase[15] is, to our knowledge, the first unambiguous crystallographic result in this field. Since then, a 2.7 Å structure has been published of *Fusarium oxysporum* endoglucanase I complexed with a nonhydrolyzable substrate analogue at sites −2, −1, and +1,[24] for which a similar distortion of the −1 sugar bound was inferred as observed in the 2.0 Å resolution chitobiose−chitobiase complex.[15]

Furthermore, our results demonstrate the involvement of the C2 substrate side chain in the mechanism of enzymatic glycosyl hydrolysis. Such neighboring group participation or anchimeric assistance may also shed light on the catalytic mechanisms of goose lysozyme[25] and soluble lytic transglycosylase,[26] two other enzymes which cleave β(1,4)-*N*-acetylglucosaminic linkages and which appear to lack a catalytic nucleophile on the enzyme. The stereochemistry around the anomeric C1 atom in the intermediate only allows attack at the equatorial position, thus limiting the mechanism to retention (Figure 1b). Our results are reminiscent of the utilization of the glycosyl C2−OH in the ribonuclease A nucleophilic mechanism.[27]

Methods

Crystals were grown as previously described.[11,15,28] Crystallographic complexes of enzymes were obtained by soaking substrate or inhibitor into enzyme crystals as described before.[9,11,15] For the hevamine−

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chitotetraose complex crystals were soaked for 24 h in 20 mg/mL chitotetraose in mother liquor pH 7.0, and data were collected to a resolution of 1.85 Å on the X31 beam line at DESY, Hamburg. All data were processed using DENZO and SCALEPACK.[29] The complex was refined with the program TNT.[30] The complexes of hevamine−allosamidin and chitobiase−chitobiose have been published before,[9,15] but they were re-refined with TNT, using the same dictionary of standard geometric parameters as for the hevamine−chitotetraose complex. The refinement with TNT of the Chitinase A−*N*-acetylglucosamine complex[11] allowed the modeling of a chitobiose molecule instead of a monosaccharide in the electron density.

Modeling. Catalytic domains of Chitinase A and hevamine were superimposed using "O".[31] The final superposition used 174 Cα pairs with an rms difference of 2.1 Å. To form a continuous saccharide chain, the pyranose ring atoms of the +1 sugar in chitobiase were superimposed on the +1 sugar in Chitinase A. Hence a hexasaccharide was composed, comprising −4/−3/−2 sugars from hevamine, −1/+1 from chitobiase, and +2 from Chitinase A. TNT[30] rigid body refinement of the hexasaccharide with Chitinase A was carried out to obtain the final structure. The enzyme molecule and each sugar residue were defined as rigid bodies, with the C1−O bonds free to adjust between the sugar rigid bodies. In hevamine, sugar binding site +1 is involved in crystal contacts, thus hampering a successful modeling.

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